AD_____

Award Number: DAMD17-99-1-9249

TITLE: Functional Sites of the erbB-2 Receptor and Its Activator Heregulin

PRINCIPAL INVESTIGATOR: Joanna E. Mroczkowska-Jasinka, Ph.D.

CONTRACTING ORGANIZATION: University of California at Berkeley
E. O. Lawrence Berkeley National Laboratory
Berkeley, California 94701

REPORT DATE: August 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Annual Summary (1 Aug 99 - 31 Jul 02)

4. TITLE AND SUBTITLE
Functional Sites of the erbB-2 Receptor and Its Activator Heregulin

5. FUNDING NUMBERS
DAMD17-99-1-9249

6. AUTHOR(S)
Joanna E. Mroczkowska-Jasinka, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California at Berkeley
E. O. Lawrence Berkeley National Laboratory
Berkeley, California 94701

E-Mail: JEMroczkowska@lbl.gov

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Over expression of the *erbB-2* (HER-2/neu) receptor occurs in up to 30% of human breast cancers and correlates with aggressive disease and poor prognosis for therapy and survival. The growth factor heregulin (HRG) binds to *erbB-3* or *erbB-4* receptors, promotes dimmer formation with *erbB-2* and induces autophosphorylation and activation of *erbB-2* signaling. It is generally accepted that HRG and *erbB-2* do not interact directly. Depending on its concentration HRG can either inhibit or stimulate cell proliferation in cell lines that overexpress *erbB-2*. This suggests some type of direct interaction between HRG and *erbB-2*. Solution structure of HRG and other data support the existence of a low-affinity binding site within the EGF-like domain of HRG. **The goal of the proposed experiments is to define the predicted sites of interaction between HRG and the *erbB-2* receptor, through generation of HRG and *erbB-2* deletion mutants.**

During the third year of funding, I continued the experiments according to the data obtained in the previous years: a) performed proliferation assays using Ba/F3 cells infected with deletion mutants of *erbB-2* as well as autophosphorylation studies. I also infected with *erbB-2* deletion mutants 184B5 immortalized human epithelial cells in order to establish a better *in vitro* system to study *erbB-2* induced tumorigenesis; b) continued to work on purification of recombinant proteins containing alanine substitution in heregulin □1 gene and generated cell lines expressing heregulin mutants in cells coexpressing different levels of erbB receptors. The main result of my work was generation of 8 cell lines expressing the *erbB-2* receptor containing different deletions in the extracellular domain, which allowed me to map functional site of this receptor, and 15 cell lines with different levels of erbB receptors expressing heregulin with alanine substitutions within the EGF-like domain. These cell lines will be used in the experiments designed to determine the mechanism of interaction between *ErbB-2* and heregulin.

14. SUBJECT TERMS
breast cancer, erbB-2 receptor, HRG

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

5. INTRODUCTION

The epidermal growth factor (EGF) receptor family comprises four transmembrane tyrosine kinases (EGFR, *erbB-2*, *erbB-3* [kinase defective], and *erbB-4*) that are involved in the genesis and progression of a variety of human carcinomas. Amplification and overexpression of the *erbB-2* (HER-2/neu) receptor tyrosine kinase receptor, occurs in up to 30% of human breast cancers and has been shown to correlate with aggressive disease and poor prognosis. Despite the involvement of *erbB-2* in tumor development, its exact biological functions have yet to be elucidated. Heregulin (HRG, NDF) is a 45kDa growth factor that is expressed in about 30% of breast carcinomas and is associated with invasion and metastasis. HRG was initially isolated as a specific ligand for *erbB-2*, however it is generally accepted that HRG does not bind directly to *erbB-2*. HRG does bind to *erbB-3* and *erbB-4* with high affinity, which promotes heterodimerization with *erbB-2*, inducing autophosphorylation and activation of *erbB-2* signaling.

It was recently found that HRG does have an extremely low affinity for *erbB-2*. The solution structure of HRG indicates an EGF-like domain of HRG with two clusters of amino acids apparently involved in receptor binding: a conserved high-affinity binding domain of 35-40 amino acid residues located in the N-terminal portion, and a region of non-conserved amino acids comprising a low-affinity binding domain in the C-terminal portion. HRG-neutralizing antibodies prepared in Dr. Lupu's laboratory directed against the C-terminus (α1) and the N-terminus (α3) blocked HRG-induced growth effects. However, the antibodies failed to block *erbB-2* receptor phosphorylation when used separately; blockage of receptor phosphorylation was observed only when the antibodies were used in combination. These observations prompted us to hypothesize that the C-terminus constitutes a site for low-affinity binding between HRG and *erbB-2*.

The extracellular region of *erb-B* receptors has been organized into a four-domain model, in which subdomain III contributes most of the determinants involved in ligand binding and signal transduction. To determine the putative site involved in the interactions between *ErbB-2* and growth factors, a number of synthetic peptides with sequences homologous to specific *erbB-2* regions (termed the RL series) were generated in Dr. Lupu's laboratory. It was shown that the peptide RL2, which was derived from a sequence in the *erbB-2* extracellular domain, was capable of specifically blocking HRG-induction of *erbB-2* tyrosine phosphorylation. Due to this fact, it is possible that this region (in the proximity of the RL2 peptide) constitutes a critical region of the *erbB-2* receptor responsible for HRG induction of *erbB-2* heterodimerization and activation.

The goal of this project is to explore the therapeutic potential of HRG for use against breast cancers, which overexpress *erbB-2*, through the:

a) **Generation of HRG mutants that will exclusively bind to *erbB-2* and**
b) **Identification of the *erbB-2* functional site (site responsible for receptor heterodimerization).**

Identification of the specific site of interaction between HRG and *erbB-2* as well as identification of the *erbB-2* site responsible for HRG induction of receptor heterodimerization will enhance the necessity of developing HRG-targeted agents, including HRG antagonist and/or HRG-targeted vehicles that could improve the delivery of chemotherapeutic agents.

6. BODY

The goal of the research in this proposal is to extend the ongoing studies. The following experiments are designed to shed light on the biological and molecular mechanisms by which heregulin can induce the activation of the *ErbB-2* signaling pathway of epithelial cells that leads to the aggressive forms of tumors. The original technical objectives were as follows:

- **Task 1: To generate deletion mutants of the *erbB-2* extracellular domain in order to determine the site of the *erbB-2* receptor that allows HRG induction of *erbB-2* tyrosine phosphorylation and/or its heterodimerization with other members of the *erbB* receptor family.** These studies are being accomplished by generating a series of *erbB-2* deletion mutants and subsequent infection of the obtained constructs in a retroviral vector into the pro-B-lymphocyte cell line Ba/F3. Following the transfection the cells will be tested for their ability to grow and proliferate in the presence or absence of the wild type HRG.

- **Task 2: To generate HRG deletion mutants (Δ-HRG) and determine the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling.** I will generate two sets of heregulinβ mutants. One at the N-terminal domain, which contains the low-affinity binding site involved in the direct type of interaction with *erbB-2* and a second at the EGF-like domain which binds to *erbB-3*, and stimulates its dimerization with other members of the receptor tyrosine kinases family. These studies will be accomplished by creating partial deletions within critical domains and subsequent point mutations to determine which amino acids are critical for interactions with the receptor. The Ba/F3 pro-B-lymphocyte cell line transfected with the *erbB* receptors will be treated with wild type and mutant HRG in order to test if the *erbB-2* signal transduction pathway is active.

STATEMENT OF WORK:

Task 1: To establish cell lines expressing *erbB-2* receptor with deleted part of the extracellular domain, in order to determine the site of the *erbB-2* receptor that allows HRG induction of *erbB-2* tyrosine phosphorylation and/or its heterodimerization with other members of the *erbB* receptor family.

ACCOMPLISHED OBJECTIVES FROM INITIAL TASK 1 AND 2:

A) *Determination, by proliferation assays, of the functional site of the erbB-2 receptor that allows HRG induction of erbB-2 tyrosine phosphorylation* (Months 24-32)

Determination of the functional site of the *erbB-2* receptor was performed in an interleukin-3 (IL-3) dependent Ba/F3 pro-B-lymphocyte cell line. These cells, when cultured without IL-3 can survive with heregulin when the functional *erbB-2* receptor is expressed. Although many different approaches were taken to complete this task none of the assays has proven sensitive enough in order to be conclusive. We were able to measure different survival ratio in the cells expressing *erbB-2* wt when the cells were cultured with or without heregulin (Fig. 1). However, none of the assays was sensitive enough to measure any significant difference in the cells expressing receptors with a deletion within the extracellular domain (Fig 2). This prompted us to look for a new and better model for our studies.

B) *Determination of the functional site of the erbB-2 receptor that allows HRG induction of erbB-2 phosphorylation by phosphorylation and immunoprecipitation* (Months 18-24)

To determine the functional site of the *erbB-2* receptor that allows HRG induction of receptor phosphorylation, we analyzed the difference in the phosphorylation pattern of the receptor in Ba/F3 cells, expressing various *erbB-2* mutants, treated with heregulin and these not treated. This part of the task appeared to be more complicated than we originally expected. This complication was due to the fact that some of selected Abs did not bind specifically to the proteins of interest. It was therefore necessary to test a number of new antibodies and to optimize the conditions in order to detect any changes in the phosphorylation profile of the receptor. In order to increase binding specificity and decrease observed background we tried also to immunoprecipitate *erbB-2* from total cell lysate with specific anti-*erbB-2* Ab but this strategy was not successful either. Although we have taken many attempts we were not able to eliminate background from our western blots performed to detect phosphorylated form of the receptor (Fig. 3). This was another reason why we decided to look for a better model for our studies.

After extensive literature search we have chosen HMEC (human mammary epithelial cells) 184B5 cells. This cell line was obtained from reduction mammoplasty tissue and further immortalized by benzo[a]pyrene. Moreover, it was shown that these cells acquire more invasive and metastatic phenotype after being transfected with wt *erbB-2* receptor than the parental cell line. Therefore we infected these cells with the generated *erbB-2* mutants containing deletions within extracellular domain. Established cell lines expressing the

introduced variants of *erbB-2* receptor were used for further experiments. Cells were treated and protein lysates were analyzed as described above. Western blot analysis has shown high expression level of *erbB-2* receptors, however we were not able to show by WB any difference in phosphorylation pattern due to the high background. In order to reduce the background we preformed immunoprecipitation as described above and subsequently equal amounts of protein were separated on SDS PAGE gel. The proteins were transferred to nitrocellulose membranes and incubated with specific antibodies against *erbB-2* receptor and anti-phosphotyrosine to detect activated form of *erbB-2* receptor. This attempt turned out to be very successful, on the blot incubated with anti-phosphotyrosine Ab we could observe very strong and specific bands corresponding to the positions of *erbB-2* wt and *erbB-2* deletion mutants number 3 and 4 as well (Fig. 4). This experiment allowed us to map functional site of *erbB-2* receptor involved in dimmer formation and autophosphorylation within 20 amino acids of the extracellular domain of the receptor. The results demonstrate that region within 415-439 aa is crucial for dimmer formation and/or stabilization and induction receptor phosphorylation.

Task 2C: To determine the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling. Months 24-32: To perform anchorage-dependent and –independent proliferation assays.

In order to test whether or not overexpression of the functional *erbB-2* receptor results in more aggressive cell growth and invasive phenotype we decided to test cells ability to grow in anchorage independent conditions. $2x10^4$ cells were suspended in 1 ml of 1,5% methylcellulose solution in MGEM, seeded onto 6-well plates coated with PolyHEMA. Cells were fed once a week and examined after 3 weeks of culture. Unfortunately in this assay we were not able to show any effect of *erbB-2* on cell invasiveness. Although *erbB-2* transfected B5ME cells formed colonies under these conditions we did not observe any colony formation neither, with the cells infected with wt *erbB-2*, neither with the cell expressing functional *erbB-2* receptor (Fig. 5). Nevertheless, we tried alternative approach and looked for phenotypic changes in cells grown in matrigel 3D culture. 10^4 cells were suspended in 0.15 ml of matrigel, seeded onto 12-well plates coated with matrigel. Cells were cultured for 10 days and examined under microscope. We were not able to see any differences under these conditions either (Fig. 6). These observations may suggest that overexsspression of *erbB-2* alone is not sufficient in normal cells for transformation and tumorigenesis. We think that the *erbB-2* effect on B5ME cells phenotype observed earlier was related rather to the method used for establishing this cell line than to *erbB-2* itself.

Task 2D: To express mutated HRG recombinant protein for further studies of the ability of these mutants to lose/retain HRG's ability to induce *erbB-2* tyrosine phosphorylation and/or signaling. Months 8-14: To express HRG recombinant protein with point mutations.

Expression of the mutated heregulinβ2 recombinant protein: Initially for HRG expression I decided to use the pMAL™ Protein Fusion and Purification System because of the following advantages:

- This system has been proven to give high-level of expression of the cloned sequences.
- It enables one-step purification of the fusion protein using MBP's affinity to maltose.

However, after screening a large number of clones in order to select the clones that would be able to produce recombinant protein after IPTG induction I was unable to isolate any IPTG inducible clones. I conclude that this system was not optimal for heregulin isolation as we were able to induce expression of other recombinant proteins and the positive controls included in this experiment gave us high level of induction. Therefore I decided to use BAC-TO-BAC baculovirus expression system for the following advantages:

- The BAC-TO-BAC System is an eukaryotic expression system which generates recombinant baculovirus by relying on site specific transposition in *E. coli* to make the recombinant baculovirus (instead of homologous recombination in insect cells). Expression of cloned gene is driven by the highly active polyhedrin promoter and therefore it can represent up to 25% of the cellular proteins in infected insect cells.
- This system enables to generate viruses, which will express unfused recombinant proteins.
- This system enables protein purifiacation by affinity column using His-tag.

To express recombinant heregulin wt and mutants containing alanine substitutions in EGF-like domain we subcloned hrg cDNA from pMAL plasmid into pFastBac vector and single clones containing insert in right orientation were isolated. After transformation into competent DH10Bac™ *E.coli* cells HRG constructs underwent transposition resulting in recombinant bacmid DNA. Recombination was confirmed by PCR (Fig. 7) and positive clones were chosen for transfection into insect cells and further purification. This task is currently in the process of being completed.

Task 2E: To determine if the C-terminal domain of HRG has a low-affinity binding site that is specific and unique for *erbB-2*. Months 16-24: To perform phosphorylation assays to test HRG mutants for their ability to induce tyrosine phosphorylation of the *erbB* receptors.

Due to the difficulties with obtaining and purifying heregulin recombinant proteins containing alanine substitutions within EGF-like domain we decided to try alternative approach in parallel with protein purification. We decided to infect with heregulin mutants breast cancer cell lines described to co-express different levels of *erbB* receptors in order to study the effect of endogenous heregulin on cell growth and phenotype. For this experiment we used SkBr-3 (EGFR, ErbB-2 +++, ErbB-3-, ErbB-4-), MDA-468 (EGFR, ErbB-3, ErbB-4), BT-474 (EGFR, ErbB-2+++, ErbB-3+/-, ErbB-4+/-) cell lines.

Cloning of the heregulinβ2 mutants into pBabe retroviral vector: To clone the heregulinβ2 mutants into pBabe vector we preformed PCR using hrgβ2 cDNA containing alanine substitution within EGF-like domain as a template and specific primers derived from the heregulin sequence. Specific products of PCR were analyzed by agarose gel electrophoresis, purified from the gel and cloned into pBabe vector. After transformation into DHα5 *E.coli* cells single clones were analyzed in order to confirm presence of the insert. By sequence analysis, I confirmed that all desired point mutations were present and that other unexpected mutations have not occurred during PCR amplifications.

Infection of the cells with *HRG* mutants: To infect the cells with the hrg containing alanine substitutions within its extracellular domain, the packaging cell line TSA54A was used. The TSA54A cells were transfected with DNA constructs in order to obtain the functional virus capable of infecting breast cancer cells. Virus was collected 48h after transfection, filtered and used for infection. 24h post-infection cells were cultured with puromycin in order to select infected cells. The obtained cell lines are still under selection and/or expansion stage but first observations are very promising and as soon as this part is completed expression analysis will be performed as well as proliferation assays.

TASKS REMINING TO BE PERFORMED

Task 1: To determine the site of the *erbB-2* receptor that allows HRG induction of *erbB-2* tyrosine phosphorylation. Months 32-36: To perform receptor binding and ligand binding assays.

7. KEY RESEARCH ACCOMPLISHMENTS

- I have successfully generated eight 184B5 cell lines expressing *erbB-2* receptor with different deletions localized in subdomain III of the *erbB-2* extracellular domain.

- Using these cells I was able to map the functional site of erbB-2 receptor within 20 amino acids of its extracellular domain – this site within 415-439 aa can be a potential target for breast cancer therapy.

- I have successfully cloned the cDNA of hrgβ2 containing alanine substitution within EGF-like domain into Bacmid DNA, recombinant proteins are being purified and isolated for further experiments.

- I have successfully infected with heregulin cDNA containing five different point mutations within EGE-like domain 3 different cell lines (SkBr-3, BT 474, MDA-468) co-expressing different levels and combination of *erbB* receptors in order to establish cell lines for *in vitro* studies of interactions between *erbB-2* and heregulin. As a result I have 15 cell lines with different context of *erbB* receptors expressing heregulin with alanine substitution within EGF-like domain.

8. REPORTABLE OUTCOMES

- Establishing 184B5 cell lines expressing *erbB-2* receptor with different deletions at the putative functional site.
- Mapping the functional region of *ErbB-2* receptor involved in its activation.
- Establishing cell lines: SkBr-3, BT-474, MDA-468 expressing heregulin with alanine substitution in positions involved in interactions with *erbB* receptors.

9. CONCLUSIONS

The original goal of this proposal was to generate deletion mutants of the erbB-2 and heregulin for subsequent studies on their biological role in breast cancer.

- We have successfully infected the generated deletion mutants of the erbB-2 into the 184B5 cells. All obtained mutants express introduced gene on RNA and protein level.
- We were able to detect phosphorylated form of *erbB-2* receptor in cells expressing wt *erbB-2*, mutant 3 and mutant 4. These mutants had deletions corresponding to 439-490 aa and 461-490 aa respectively. All other mutants lost their ability for phosphorylation. This allowed us to map the functional site of the receptor within 20 amino acids of its extracellular domain. The extracellular region between 415 and 439 aa of *erbB-2* receptor can be a potential target for breast cancer therapy.
- We have successfully infected heregulin mutants containing alanine substitution within EGF-like domain into breast cancer cell lines coexpressing different level of *erbB* receptors.
- We have successfully recombined the cDNA of heregulin with point mutations within EGF-like domain with the Bacmid DNA in order to purify recombinant protein by using Baculovirus system.

These experiments will enable the further studies of interactions between *erbB-2* and HRG. The future work of Dr. Lupu's laboratory is aimed to define the functional sites involved in the cross talk between these proteins. Better understanding of this process can provide new strategies, which can help to stop or to slow down breast cancer progression.

10. REFERENCES

Not Applicable

11. APPENDICES

Appendix I: Figures and Figure legends

Figure 1. Baf3 cells survival after replacing IL-3 by HRG.

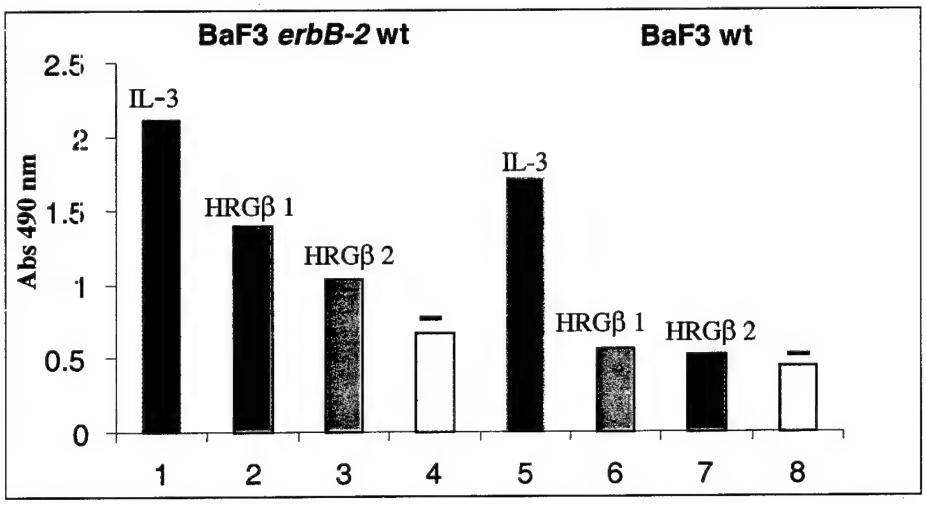
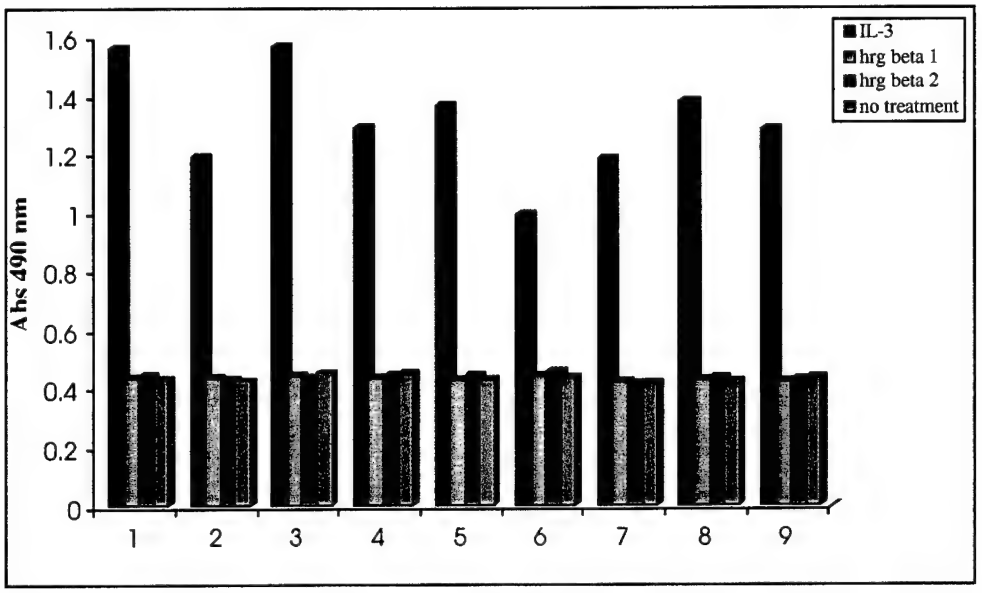


Figure 2. Baf3 cells infected with ErbB-2 constructs containing deletions lose their ability to survive when IL-3 is replaced by HRG. 1-8 cells infected with the ErbB-2 deletion mutants 1-8 respectively, 9 cells infected with an empty vector.



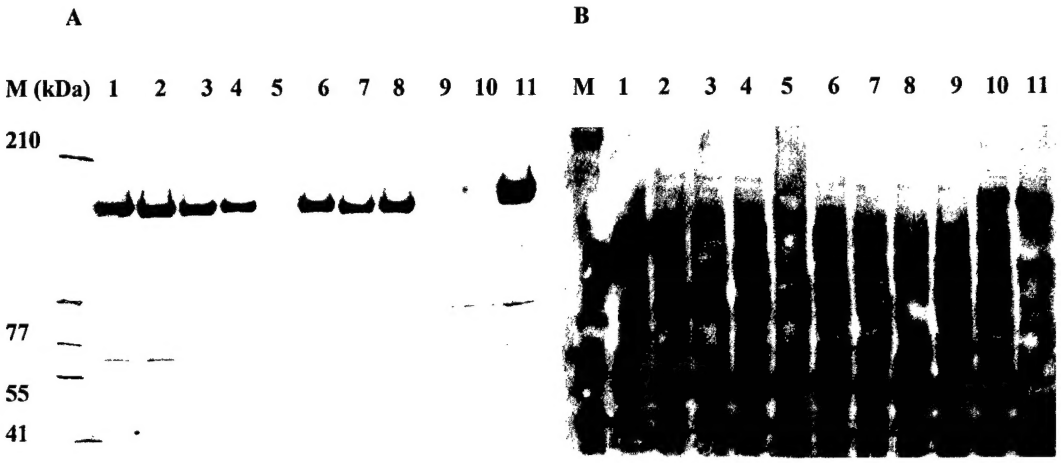


Figure 3. Western Blot to check the expression of erbB-2 mutants in BaF3 cells. Baf3 cells infected with ErbB-2 constructs were collected and lysed with Ripa buffer. Cell lysates were separated on PAGE gels, transferred and blotted with anti-ErbB-2 (A) and anti-phosphotyrosine (B) Abs. Numbers 1-8 correspond to the subsequent mutants; 9. Cells infected with an empty vector, 10. Cells infected with wt erbB2, 11. MB-MDA 453 cells –positive control.

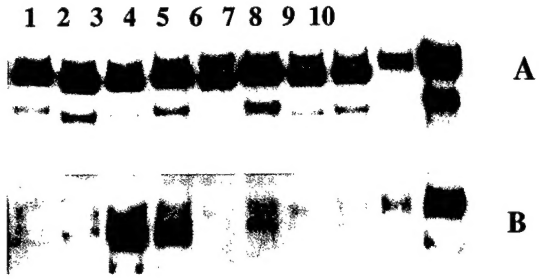


Figure 4. Western Blot to check the expression of erbB-2 deletion mutants in 184Bcells. 184B5 cells infected with ErbB-2 constructs were collected and lysed with Ripa buffer. Cell lysates were immunoprecipitated with anti-ErbB-2 Ab, separated on PAGE gels, transferred onto nitrocellulose membrane and incubated with anti-ErbB-2 (A) and anti-phosphotyrosine (B) Abs. Numbers 1-8 correspond to the subsequent mutants; 9. Cells infected with an empty vector, 10. Cells infected with wt erbB2;

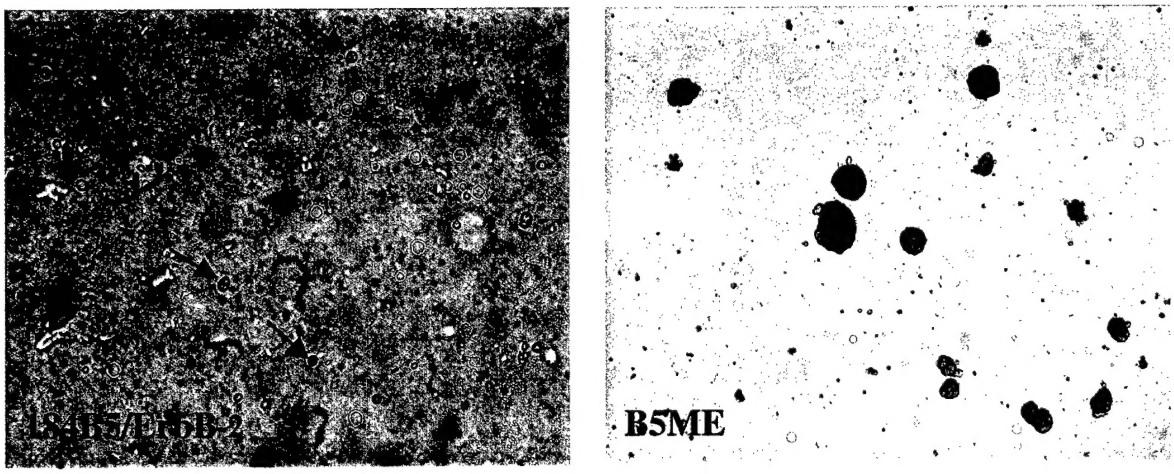


Figure 5. 184B5 cells overexpressing ErbB-2 do not acquire ability for anchorage independent growth. 20K cells were suspended in 1 ml of a 1.5% methylcellulose solution in MGEM, seeded onto 6-well plates coated with PolyHEMA. Cells were fed once a week and pictures were taken after 3 weeks of culture. More aggressive B5ME cells are able to divide and grow under this condition and form big cell clusters. 184B5 cell infected with functional ErbB-2 receptor are not able to grow in anchorage independent manner.

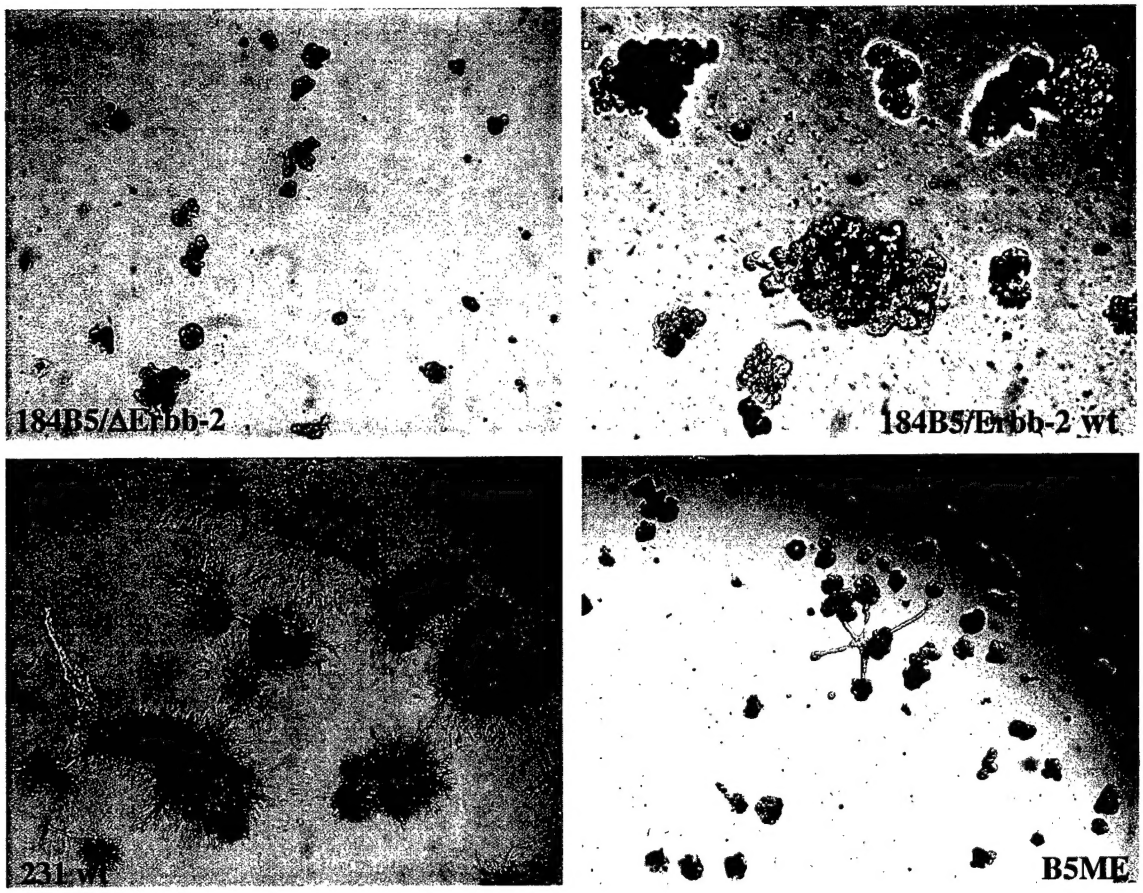


Figure 6. 184B5 cells overexpressing ErbB-2 do not have growth advantage in matrigel. 10K cells were suspended in 0.15 ml of matrigel, seeded onto 12-well plates coated with matrigel. Cells were cultured for 10 days and pictures were taken. 231 and B5ME cell lines were used as a positive control in this experiment.

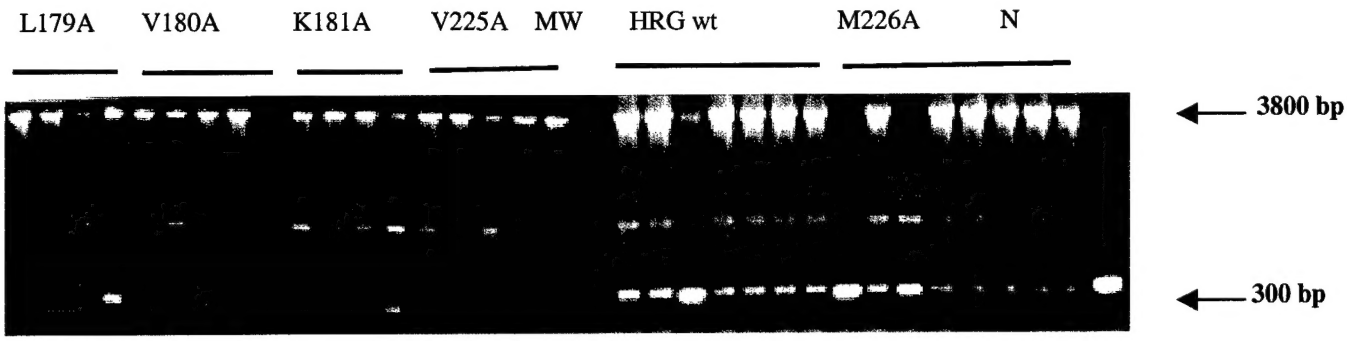


Figure 7. Test to check the efficiency of recombination between HRG cDNA and BACMID cDNA. For each construct few colonies were picked and analyzed: plasmid DNA was isolated from white single clones obtained after recombination and PCR was performed. Products obtained by PCR were separated by electrophoresis. 300bp product corresponds to empty bacmid plasmid and 3800bp product correspond to bacmid containing recombinant HRG DNA. For each point mutation designed in HRG there were positive BACMID clones containing recombinate HRG cDNA. N is a negative control DNA isolated from the bacmid plasmid.